Ligation and Transformation Protocol

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1A. Solutions and Reagents for Ligation

1. Filtration units should be prerinsed to remove Tween-20 present in the membranes as a wetting agent. Microfuge tubes should be siliconized and the use of aerosol resistant tips is strongly recommended.
2. **10x Potassium Glutamate Buffer (KGB)**[1] consists of 1 M potassium glutamate, 0.25 M Tris acetate, pH 7.6, 0.1 M magnesium acetate, 5 mM □□mercaptoethanol and 500 µg/mL BSA. Combine 3.33 mL of 3 M potassium glutamate, 625 µL of 4 M Tris acetate, pH 7.6, 1 mL of 1 M magnesium acetate, 3.5 µL of 14.3 M □□mercaptoethanol, 500 µL of 10 mg/mL BSA and 4.54 mL of Type I water. Prepare 1 mL aliquots and store at -20°C. A salt/magnesium gradient will occasionally form if the tube is frozen. Mix well before use. A working tube may be kept at 4°C for over 1 year.
3. **5x Tris Acetate Ligation Buffer** (TALB) is recommended for sticky end ligations and consists of 0.165 M Tris acetate, pH 7.6 at 25°C, 0.33 M potassium acetate, 50 mM magnesium acetate, 500 µg/mL BSA, 5 mM ATP, 0.05% NP-40 and 2.5 mM DTT.[2] The inclusion of NP-40 is useful for situations in which there are extremely limited amounts of DNA available, and can double the number of transformants in such cases by preventing DNA from adhering to the tube walls (data not shown). DTT is added as a separate component, as 100 mM magnesium tends to precipitate out when combined with 5 mM DTT. Combine 165 µL of 1 M Tris acetate, pH 7.6, 330 µL of 1 M potassium acetate, 50 µL of 1 M magnesium acetate, 50 µL of 10 mg/mL acetylated BSA, 50 µL of 1% NP-40, 50 µL of 100 mM ATP and 305 µL of Type I water. Aliquots are stable indefinitely at -20ºC in the absence of DTT. Do not forget to add 1 µL of 100 mM DTT to each 20 µL ligation reaction. (Similar buffers are commercially available, without ATP or NP-40, as New England Biolabs Restriction Buffer 4, Fermentas Y/Tango, Roche Molecular Buffer A, etc.)
4. **1 M Tris acetate, pH 7.6**: Method 1: Dissolve 18.12 g of Tris acetate in 80 mL of Type I water. Adjust the pH to 7.6 with NaOH and bring the volume to 100 mL with Type I water. Be sure that the pH probe is Tris-approved. Filter sterilize. Method 2: Dissolve 12.114 g of Tris base in 80 mL of Type I water. Adjust the pH to 7.6 with glacial acetic acid and bring the volume to 100 mL with Type I water. Filter sterilize. The optimum pH for T4 DNA ligase is 7.2-7.8. Tris buffer that is pH 7.6 at 22°C is pH 7.35 at 14°C.
5. **1 M potassium acetate**: Dissolve 19.63 g of potassium acetate in 80 mL of Type I water. Adjust the volume to 100 mL and filter sterilize.
6. **1 M magnesium acetate**: Dissolve 21.446 g of magnesium acetate in 80 mL of Type I water. Adjust the volume to 100 mL and filter sterilize.

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1. McClelland, M., et al. 1988. *Nucleic. Acids Res.* 16, 364.
2. Modified from O'Farrell, P. et al. 1980. *MGG* 179, 421.

7. **5x Blunt End Ligation Buffer** (BRL Ligation Buffer):[3] consists of 250 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) PEG 8000. To prepare 10 mL of buffer, weigh 2.5 g of PEG 8000 in a 15 mL Falcon 2097 tube that has been treated with antistatic spray or wiped with a sheet of fabric softener. Microwave a 100 mL bottle of Type I water for 1 minute on High. Add 4.4 mL of hot water and 2.5 mL of room temperature 1 M Tris-HCl, pH 7.6 to the PEG and immediately mix to dissolve. Cool the mix to room temperature and add 500 µL of 1 M $MgCl_2$, 500 µL of 100 mM ATP and 50 µL of 1 M DTT to a final volume of 10 mL. Aliquot for storage at -20°C. Failure to heat the water will result in PEG that will take approximately 24 hours to dissolve.

1B. Cloning

1. A caveat on dephosphorylation: the most common reason for failure to obtain colonies is a result of adding too much BAP or CIP to the vector prep. These enzyme preparations are difficult to purify and are frequently contaminated with exonucleases and phosphodiesterases. BAP and CIP are routinely used at elevated temperatures because the contaminants are less active at high temperatures. Excess enzyme will introduce contaminating exonucleases and nibble away restriction overhangs. In addition, BAP is more heat-resistant and is difficult to completely inactivate. Precise determination of the concentration of 5' ends is required. Calculate the exact number of ends: 2 x (g of DNA)/[size in bp x (660 Da/bp) = moles of ends of a double-stranded DNA. Use exactly enough BAP or CIP for the number of ends. This usually requires a significant dilution of the stock. Excess CIP is also reported to inhibit complete dephosphorylation. Because the byproducts of the reaction inhibit the reaction itself (dephosphorylation generally only proceeds to 95% completion), I then clean up the DNA by adding 10x stop mix consisting of 200 mM EGTA, pH 8.3, 10x TNE100 (1x TE containing 100 mM NaCl) and 10% SDS and heat inactivating at 56-68°C for 15-45 minutes; followed by phenol:chloroform extraction and ethanol precipitation. Then I repeat the entire CIP protocol all over again to dephosphorylate the residual molecules that failed to dephosphorylate the first time. This has proven to be infinitely safer than using too much enzyme. SAP is thermally sensitive and must be used at 37°C, but lacks the contamination problems of BAP and CIP. Generally, one unit of SAP is required for the complete removal of terminal phosphates from 100 pmol of 5' ends of DNA. I have switched to the exclusive use of SAP, but supply these notes on the use of BAP and CIP for those who wish to use these enzymes.
2. **Vector prep**: combine 25 µg of vector (for instance, 25 □g of pUC9 equals 14.4 pmol; enough for 28 ligations using 0.5 pmoles per ligation reaction) with 20 µL of 10x KGB, 80 units @ of *BamH* I and *Hind* III, 3 units of Shrimp Alkaline Phosphatase and Type I water to a final volume of 200 µL. Incubate at 37°C for 3.25 hours. Small scale reactions using single restriction enzymes should be performed and analyzed with uncut plasmid DNA and 5 µL of the vector digest on an analytical 0.8% agarose gel to confirm that both restriction enzymes are performing as expected.

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[3]. BRL Technical Bulletin 5224-1 1992.

Bromphenol blue runs at 800-1000 bp on an 0.8% gel. You will need to run the samples far enough to distinguish single-cut 2665 bp pUC9 from double-cut 2645 bp vector. Stain agarose gels in 1.0 mg/mL ethidium bromide or 1x SYBR Gold (Molecular Probes) for 15 minutes (no destain necessary) and visualize on a Fuji ImageReader (Fuji Medical) or photograph the gel on a UV-transilluminator (Fotodyne, Hartland, WI) with Type 667 film (Polaroid, Cambridge, MA). A typical EtBr exposure is f/5.6 for 1 1/2 sec with Kodak type 15 and type 22 filters. Rinse the transilluminator filter with water and discard gloves, KimWipes and the gel as ethidium bromide waste. Note that this is an analytical gel only.

3. It may be helpful to purify the vector by spin column chromatography or 30K MWCO cartridge to eliminate the polylinker fragment. It is not necessary to gel-purify the vector if the only cuts are in the polylinker cassette. Should you desire to do so in order to feel better, hand stain the gel in the dark with 10 mL of 1 mg/mL stain rather than use a staining bath container that is almost certainly contaminated with other plasmid DNA as well as nucleases from contaminating bacteria and molds. DNase activity in the solution can make it difficult to isolate clonable DNA from gels stained in contaminated baths.
4. **Insert prep**: combine 14.4 pmol of insert (PCR product or plasmid-derived) with 20 μL of 10x KGB, 80 units @ of *BamH* I and *Hind* III and Type I water to a final volume of 200 μL. Incubate at 37°C for 3.25 hours. Small scale 20 □L reactions using single restriction enzymes should be performed and analyzed with uncut insert and 5 μL of the insert digest on an analytical native 12% acrylamide gel with 20/100 bp markers (GenSura) to prove that both restriction enzymes are performing as expected. Run the gel using constant watts appropriate for the gel box. You will need to run the samples far enough to distinguish single-cut insert from double-cut insert. Stain the gel in ethidium bromide or SYBR Gold for 10 minutes (no destain) and photograph the gel on a UV-transilluminator. If ANY primer dimer contaminates the PCR reaction, the PCR fragment of correct size MUST be purified an a native 8% polyacrylamide gel prior to cloning. Passively elute or crush and soak the band of interest in 300 mM sodium acetate. Gel slices from 1-cm wells are soaked in 330 μL of NaOAc in a siliconized microfuge tube, while slices from a 1-inch are soaked in 1000 μL. Place tubes on a room temperature rotator for at least 4 hours or overnight. Ethanol precipitate the eluted DNA with Linear PolyAcrylamide (LPA) carrier. Digestion of the PCR fragment will typically generate 2 smaller bands equivalent to single- and double-cut fragments. As long as there is no uncut fragment visible on an ethidium bromide stained native polyacrylamide gel, the material is suitable for cloning.
5. If restriction digestion of the insert or vector results in multiple DNA fragments, then gel-purification is mandatory.
6. While the analytical gels are being run, clean up both the insert and vector by extracting twice with Tris-buffered phenol:chloroform, once with chloroform and then ethanol precipitate with LPA carrier. Do not use tRNA carrier, since it will inhibit the ligation. If either of the preparations require further restriction digestion, this step is much more efficient when the DNA is pre-cleaned. Repeat the set-up as described above and cut for approximately one hour. If one of the enzymes is not performing, consider switching to a new lot.

7. **Ligation**: mix 0.5 pmol of *BamH* I/*Hind* II-cut dephosphorylated pUC vector with 5 pmol of *BamH* I/*Hind* III-cut insert, 2 µL of 5x TALB, 1 µL of 100 mM DTT, 1 Weiss Unit of T4 DNA ligase and Type I water in a final volume of 20 µL. Incubate the reaction at 14°C for at least 3 hours or overnight. Heat inactivate the ligase at 65°C for 5 minutes. Controls consisting of 1) the reaction minus the insert and ligase, and 2) the reaction minus the insert should be run the first time a batch of *BamH* I/*Hind* II-cut dephosphorylated pUC vector is used.
8. When bi-directional insertion of the DNA fragment into the vector is desired, in order to reduce toxic effects of the insert, the *Sma* I site or *Eco*R V (or Eco32I) site (depending upon the plasmid) may be used to linearize the vector.

1C. Notes on Cloning

1. This protocol, as written, is designed for PCR fragment inserts of 80 to 100 bp in size. For other insert sizes, the suggested insert to vector ratios are as follows: fragments ≤ 1 kb uses a 2:1 or 3:1 ratio, inserts that are within 1 kb of the vector use a 1:1 ratio, while larger inserts reverse the ratio to 1 part insert:2 parts vector.
2. This protocol uses 0.5 pmols (866 ng) of pUC vector in a 20 □L ligation reaction for a final vector concentration of 43 ng/□L. While the quantity of vector can be scaled down to as little as 30 fmols (52 ng) of nucleic acid, the larger amount avoids problems that can arise from inherent errors in spectrophotometer measurements of nucleic acid concentrations or loss of material during purification.
3. PCR products should be cleaned by 30K MWCO cartridge with a 300 □L rinse with Type I water. This will remove most, but not all, of the residual dNTPs. Resuspend the PCR product in 30 □L of water and transfer to a siliconized microfuge tube. Determine the concentration spectrophotometrically and calculate the micromolar concentration. For an accurate concentration determination of small fragments, paste the sequence into the IDT (www.idtdna.com): Customer Service: Oligo Analyzer program to obtain the extinction coefficient. Extinction coefficient calculations are significantly more accurate than calculations using the assumptions of 1 A_{260} = 20 - 50 □g/mL Divide the A_{260}-A_{320} value by the extinction coefficient and multiply by the dilution factor and 1x 10^6 to obtain the µM concentration. If the yield of PCR product is greater than the concentration of primers used in the PCR reaction, assume 100% conversion to product and 85% recovery from the 30K cartridge and divide the primer concentration by the volume of sample to obtain a crude estimate of the DNA concentration.
4. If the analytical gel indicates that the PCR product is composed of a single band, Centrex 30K purification and ethanol precipitation may be sufficient. The PCR product should be carefully analyzed by analytical gel. If the slightest trace of any primer dimer contaminates the reaction, the PCR fragment of correct size must be purified on a native polyacrylamide gel prior to cloning.
5. Because there are usually 10-20 pmol of dsDNA in the restriction digest that may contain 10^{13} recognition sites, modifications of normal digestion conditions are required. If timepoint analysis shows that 80 units of enzyme in a 150 µL restriction reaction for 3.25 hours are required to completely digest 25 µg of pUC vector

(equivalent to 14.4 pmol) with *BamH* I and *Hind*III, then 14.4 pmol of PCR fragment will require the same digestion conditions.

6. Simultaneous digestion of the pUC vector with both enzymes in the presence of 3 units of Shrimp Alkaline Phosphatase (Amersham BioSciences) in potassium glutamate buffer (KGB) usually provides the best material for cloning. Sequential extended digests should be avoided due to exonuclease activity present in many lots of enzyme. *BamH* I and *Hind* III must have low levels of exonuclease activity when used under these conditions. *BamH* I and *Hind* III were chosen because they are the most compatible double digest restriction enzymes available for cloning into the polylinker. Star activity due to excessive enzyme concentration must be avoided.[4]
7. KGB may cause some distortion of fragments < 800 basepairs on agarose gels of < 1%.[5]
8. Avoid using ligation buffer that shows the slightest trace of precipitation.
9. UV-generated damage to DNA stained with ethidium bromide reduces the transformation efficiency. One minute of exposure to 312 nm UV can reduce the transformation efficiency by > 90%.[6] [7] UV-damaged DNA products include cyclobutane pyrimidine dimer (T-T), 5-thyminyl-5,6-dihydrothymine, 4,6-diamino-5-formamidopyrimidine, 5-hydroxy-5,6-dihydrothymine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 6-hydroxy-5,6-dihydrocytosine and 8,8-adenine dehydrodimers (from poly(dA). Guanosine is reported to protect DNA from damage by UV transillumination. 1 mM guanosine added to the gel and the running buffer "increased the yield of clones by a factor of about 400, compared with conventionally prepared, unprotected DNA" exposed to 312 nm UV.[8]
10. SYBR Gold is more sensitive than ethidium bromide and can be removed by ethanol precipitation.
11. Since 66 µM dATP inhibits T4 DNA ligase by 60%[9], the PCR amplified insert should be purified before being used in the ligation reaction.
12. To estimate the frequency of colonies with the correct insert, 10 µL samples from 10 to 20 cultures can be PCR amplified according to a procedure for amplification of bacterial colonies,[10] using the forward and reverse sequencing primers. Colony PCR initiated from transformation reactions can give rise to false positives if the primers anneal to the insert sequence due to excess DNA insert from the ligation reaction that is spread as part of the transformation reaction onto bacterial plates.[11] Typical ligation protocols utilize 5 pmol of insert PCR product in a 20 µL reaction (0.25

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4 B. Polisky, P. Greene, D. Garfin, B. McCarthy, H. Goodman, H. Boyer. Specificity of substrate recognition by the EcoR I restriction endonuclease. Proc. Natl. Acad. Sci. 72, 3310, 1975.

5 USB Comments 1989 16 (2) 12.

6 BioTechniques 1999, 11:747-748

7 BioTechniques 1996, 21:898-903

8 D. Grungermann and E. Schomig, 1996. Protection of DNA during preparative agarose gel electrophoresis against damage induced by ultraviolet light. BioTechniques 21(5):898-903.

9 BRL Technical Bulletin 5224-1, 1992.

10 D. Gussow and T. Clackson, Nucleic Acids Res. 17 4000 (1989).

11 Qing Dallas-Yang, Guogiang Jiang and Frances M. Sladek, 1998. Avoiding false positives in colony PCR. BioTechniques 24 (4) 580-582.

pmol/µL) with 0.5 pmols of vector. Assuming that all the vectors acquire a single copy of insert, 4.5 pmols (0.225 pmol/µL) of excess insert remain in the ligation reaction. 2 µL of a 1:5 dilution of the ligation reaction (0.09 pmol) is electroporated and diluted 1:9 with SOC media (0.01 pmol/µL). If 100 µL (1.0 pmol) is spread on a 55.4 cm^2 plate (0.018 pmol/mm^2) and a 4 mm^2 agar pick is used, then 0.072 pmols (4.35×10^{10} molecules) of background insert are available for a 100 µL colony PCR assay. To avoid this problem, at least one primer should anneal to the vector. Use of the Forward and Reverse Sequencing primers is recommended, as this allows the determination of double inserts or vector-only colonies. A negative control consisting of vector-only is also recommended for purposes of band identification.

13. Transfer 10 µL of overnight cell culture to a 650 µL microfuge tube containing 500 µL Type I water. Heat the diluted culture at 95°C for 5-10 minutes to lyse the cells, destroy proteinases and release denatured plasmid DNA. Temperature is critical; variation due to inadequate heating of differing volumes of water squirted into the heating block or a heat block that is below about 93°C will result in the failure of this technique. Use a thermistor to confirm the 95°C temperature. Microfuge at room temperature for 2-5 minutes. If a cell pellet is visible after this treatment, you added too many cells or the heating method failed to reach the correct temperature.
14. Combine 2 µL of 10x Ampli*Taq* buffer II, 0.4 µL of 10 mM dNTPs, 1 µL @ 10 µM FSP and RSP, 5 µL of cell extract, 0.5 µL of 5 Unit/□L Ampli*Taq* and Type I water to a final volume of 20 µL. Overlay the reactions with 20 □L of silicone oil (if required) and incubate in a Perkin Elmer Thermal Cycler 480, at 93ºC for 3 min, followed by 20-30 cycles of 93ºC for 30 sec, 55ºC for 60 sec and 72ºC for 1 min. The program for the Perkin Elmer 9600 is 93ºC for 3 min, followed by 20-30 cycles of 93ºC for 30 sec, 55ºC for 15 sec and 72ºC for 1 min. Analysis of 10 µL of each reaction with the GenSura 20/100 bp ladder on an 8% native acrylamide gel stained with ethidium bromide or SYBR Gold will determine which colonies contain single inserts of the correct size. If all of the analytical samples have a single insert, the remainder of the clones are assumed to have inserts also and are scheduled for sequencing. In the event of any vector-only or primer-dimer clones, all of the samples should be checked before sequencing.

2A. Solutions and Reagents for Transformation

1. Scrupulously avoid detergent contamination of all containers used in this process and carry out all processes aseptically.
2. **Luria Broth (LB):** combine 10 g of Bacto tryptone (Difco, Detroit, MI), 5 g of Bacto yeast extract (Difco) and 5 g of NaCl with 800 mL of water and adjust the pH to 7.0. Adjust the media volume to 1 liter before autoclaving. Note that you will need 4 x 500 mLs of LB in order to prepare competent cells. Store at room temperature. Do not substitute LB prepared with 10 g of NaCl, as arcing may result during electroporation.
3. **100 mL ice cold 10% glycerol**: Combine 10 mL of enzyme grade glycerol with 90 mL of Type I water. Filter sterilize through prerinsed filtration units. Autoclaving glycerol can reduce the transformation efficiency of the competent cells. Glycerol will start to decompose to ethylene aldehyde at its boiling point of 182°C.

4. Four liters of ice cold filter sterilized Type I water
5. Chilled JA10 rotor or equivalent
6. Six ice cold 500 mL sterile centrifuge bottles
7. 80-100 ice cold sterile screw-cap cryogenic vials (labeled)
8. Electroporation apparatus and ice cold 1 mm gap cuvettes
9. SOC contains twice the Bacto tryptone of LB to provide amino acids for rapid repair of cell walls damaged by the generation of competent cells and electroporation. NaCl is used to maintain isotonicity and glucose is provided for an energy source. SOC consists of 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose. Combine 20 g of Bacto tryptone, 5 g of Bacto yeast extract, 10 mL of 1 N NaCl, 2.5 mL of 1 M KCl, 5 mL 1 M $MgCl_2$, 5 mL 1 M $MgSO_4$, 10 mL of 2 M glucose and 800 mL of Type I water. Adjust the pH to 7.0 ± 0.1, adjust the volume to 1 liter and filter sterilize.
10. **100 mg/mL sodium ampicillin**: dissolve 5 g of sodium ampicillin in Type I water and adjust to a final volume of 50 mL. Filter sterilize and aliquot for storage at -20°C. A final concentration of 100 µg/mL is achieved by adding 1 mL/liter of media. Carbenicillin may be substituted at equal concentrations in order to reduce the number of satellite colonies.
11. **200 mg/mL methicillin**: dissolve 500 mg of methicillin (Research Diagnostics, Flanders, NJ) in 2.5 mL of Type I water. Filter sterilize and aliquot for storage at -20°C. A final concentration of 200 µg/mL is achieved by adding 1 mL/liter of media or agar stock. Ampicillin selection of high copy number plasmids such as pUC is more effective when methicillin is combined with ampicillin, since satellite colony formation is strongly inhibited. Do not use methicillin with wild type copy number plasmids unless you are selecting for high copy number mutations.
12. **Triphenol tetrazolium chloride (TTC)** is prepared as a 1000x stock at 10 mg/mL and can be added to a final concentration of 10 µg/mL. TTC is used to plate out the transformation reactions. Under these conditions, all of the colonies will have red cores, making detection of side-by-side colonies easier.
13. **2% (w/v) 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal)**. In a glass container, dissolve 1 g X-gal in n,n-dimethylformamide to a final volume of 50 mL. Store at -20°C. The final concentration in plates is 0.4 mg/mL. X-Gal/IPTG plates are used for re-streaking the TTC colonies prior to liquid culture to eliminate co-transformation of library sequences.
14. **100 mM isopropylthiogalactoside (IPTG)**. Dissolve 1 g IPTG in Type I water to a final volume of 42 mL. If no particulate material remains, aliquot into microfuge tubes and store at -20°C. The final concentration in plates 0.2 mg/mL.
15. **2xYT** medium consists of 1.6% Bacto tryptone, 1% Bacto yeast extract, 10 mM NaCl Combine 16 g of Bacto tryptone, 10 g of Bacto yeast extract, 5 g of NaCl in 800 mL Type I water. Adjust the pH to 7.0 and bring the volume to 1 liter. Aliquot to 100 mL and autoclave.

2B. Transformation

1. **Preparation of electrocompetent DH5α cells**: autoclave 4 baffled 1 liter flasks containing 500 mL LB. Remove a 1 mL aliquot for OD blanks. Inoculate 3 mL of

media with a few µL of frozen DH5α cells obtained by scraping the edge of the frozen cell aliquot with a sterile pipette tip. Grow the cells at 37°C for 12-16 hours at 250 rpm. Inoculate each 500 mL of LB with 500 µL of overnight culture. Remove 300 µL from each flask for an initial OD_{600} determination. Incubate the flasks at 37°C while shaking vigorously at 250-300 rpm to an OD_{600} of 0.6-0.8 (approximately 10^{10} cells/mL). Note that Sambrook and Russell[12] recommend an OD^{600} of 0.35-0.4 (approximately 10^{8} cells/mL.) Transfer the flasks to ice when the cells reach the correct optical density and hold for 15 min. Using sterile technique, transfer 330 mL of the cells to 6 ice cold sterile 500 mL centrifuge bottles. Centrifuge at 4000 x g for 15 min and discard the media. Keep the cells on ice at all times from now on.

2. Wash 1: Gently resuspend each bottle in 1 media volume filter sterilized ice-cold water. Pellet at 4000 x g at 4°C for 15 minutes.
3. Wash 2: Gently resuspend in 0.5 volumes of filter sterilized ice-cold water. Pellet at 4000 x g at 4°C for 15 minutes.
4. Wash 3: Gently resuspend in 0.5 volumes of filter sterilized ice-cold water. Pellet at 4000 x g at 4°C for 15 minutes.
5. Wash 4: Gently resuspend in 0.02 volumes of filter sterilized ice-cold water. Pellet at 4000 x g at 4°C for 15 minutes.
6. Final resuspension: gently resuspend the pellet in 0.002-0.003 volume filter-sterilized ice-cold 10% glycerol by gently swirling the liquid. This results in DNA with a very low ionic strength and high resistance. The typical transformation efficiency (in my hands) is 1.4 x 10^{10} cfu/µg of supercoiled plasmid. Aliquots of 250 µL (enough for 6 transformation reactions of 40 µL each) should be quick frozen in a dry ice/ethanol bath in microfuge tubes or cryovials and stored at -80°C until required.
7. Electroporation precautions: Do not touch the output or any part of the chamber while engaging the automatic charge and pulse switch. Do not charge the power supply to full voltage of 2500 V when using small gap cuvettes. Wear safety glasses at all times.
8. Optional: Assuming that the construction's elements contain a single *Pst* I site in the polylinker, restrict the inactivated ligation reaction with 20 units of *Pst* I in ligation buffer at 37°C for 20-30 minutes to eliminate residual phosphorylated vector that was only cut with a single enzyme during prep, allowing recircularization during ligation. The *Pst* I cut will eliminate vectors that contain a polylinker and dramatically reduce background transformants lacking the correct insert.
9. Label 2 sets of siliconized microfuge tubes and a set of Falcon 2057 polystyrene tubes. Chill the microfuge tubes and the 1 mm gap cuvettes on ice. Chill the electroporation cuvette holder in ice. Assemble pipetters and sterile Pasteur pipettes.
10. You should always include a positive control of supercoiled vector DNA. You should also include ligated and unligated vector only controls.
11. Ligation reactions must be diluted in order to reduce the conductivity of the liquid in the cuvette so that arcing does not occur. Dilute the ligation reaction 1:5 by adding 2 µL of the heat-inactivated ligation reaction to 8 µL of ice cold Type I water to one set of microfuge tubes on ice.

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[12] J. Sambrook and D. W. Russell, Molecular Cloning, A Laboratory Manual, 3rd edition, Cold Spring Harbor Press, Cold Spring Harbor, NY 1.119-1.122.

12. Thaw the required number of 250 µL aliquots of electrocompetent cells DH5α cells stored at -80°C. You will need 40 µL of cells per transformation. Thaw the cells by setting the cryovial on top of ice. As soon as the cells are thawed, place the tube upright in the ice.
13. Positive control: thaw control DNA (pUC9 monomer at 10 ng/mL = 0.004 ng/2 µL). Dilute 2:10 in water in a chilled microfuge tube. Store on ice. Prepare the required number of cuvettes and place on ice.
14. Gently aliquot 40 µL of competent cells into ice-cold microfuge tubes. Add 2 µL of the 1:5 diluted DNA to each of the following tubes: 1) vector without ligase measures the background consisting of uncut plasmid, 2) vector plus ligase determines the amount of vector that was not dephosphorylated and is able to religate, 3) vector plus ligase, restricted with *Pst* I, 4) 5:1 insert:vector ligation, restricted with *Pst* I, 5) 10:1 insert:vector ligation, restricted with *Pst* I, 6) a supercoiled plasmid DNA positive control. Store the components on ice briefly if necessary.
15. A **5:1 ratio** is sufficient if the vector and insert are cut separately (allowing removal of the polylinker fragment via a 30K cartridge) and the concentrations are accurately determined. A 10:1 ratio is recommended if the polylinker is still present in the ligation reaction. If the insert concentration is in doubt, use both ratios or increase the ratio at the risk of double inserts. Higher ratios lead to double-inserts and should be avoided.
16. Refreeze any surplus cells at -80°C in dry ice/ethanol then transfer to a -80°C freezer. Cells may be refrozen once with a 2-fold loss of transformation efficiency.
17. **Settings for electroporators** for *E. coli*: *BTX model 7200 electroporator:* 0.1 cm gap cuvette (BTX P/N 610): use 40 µL of cells at 1.3-1.5 kV/and a fixed 5-6 msecond pulse length with a field strength of 13.0-15.0 kV/cm. *BioRad electroporator:* 0.1 cm gap cuvette (BioRad 165-2089): 40 µL of cells at 1.8 kV/200 ohms/25 µF with a pulse length of about 5 mseconds 2.5 kV in a 0.1 cm cuvette results in a field strength of 25.0 kV/cm and may lead to arcing. *Gibco BRL Cell-Porator:* 0.15 cm gap cuvette: use 20-25 µL of cells and 2.4 kV, 4000 ohms/330 µF with a pulse length of about 5 seconds. 2.4 kV in a 0.15 cm cuvette results in a field strength of 16 kV/cm.
18. Immediately after pulsing, add 9 volumes of room temperature SOC to the cuvette and gently transfer the cells to a Falcon 2057 tube. Seconds count. Incubate at 37°C and 250 rpm for 60 min. Plate out multiple 20, 50 and 100 µL aliquots on LB agar supplemented with 100 µg/mL sodium ampicillin, (optional 200 µg/mL methicillin), 0.4 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) and 0.2 mg/mL isopropylthiogalactoside (IPTG). Triphenyltetrazolium chloride (TTC) can be used at a significant savings to replace the X-gal/IPTG mix if blue/white selection is not required. Typically, several thousand colonies are generated.
19. Small inserts do not generate a definitive X-gal signal. Those colonies should be picked regardless of their color and re-streaked on X-gal plates in order to eliminate problems arising from co-transformation or isolation of two colonies during the initial colony picking step. Three milliliters of 2xYT medium supplemented with 100 µg/mL sodium ampicillin is inoculated from the restreaked colonies and incubated overnight at 250 rpm at 37°C.

20. A 1.5-mL aliquot of the overnight cell culture is transferred to a microfuge tube for plasmid purification via modified alkaline lysis protocols designed to produce suitable templates for fluorescent sequencing.

2C. Notes on Transformation

1. Bacto tryptone and yeast extract can cause allergic reactions.
2. All containers used to handle the bacteria (except the tubes used for the final aliquots) should be rinsed in Type I water before use.

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Please cite the sources if these procedures are used in any publication